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Nucleic Acid Polymerases of the Developing Chicken Embryo: A DNA Polymerase Preferring a Hybrid Template*

(ribonucleotide·deoxyribonucleotide hybrid)

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ABSTRACT **This paper presents a preliminary survey of the nucleic acid polymerases of the developing chicken embryo, especially of the 4-day stage. The predominant activity is that of a DNA polymerase preferring a DNA–RNA hybrid as the template. The enzyme, which is activated by Mn^{2+} ions and inhibited by *p*-chloromercuriphenylsulfonate, copies preferentially the ribo strand of a hybrid, such as poly(rA)·poly(dT), but is relatively inactive with all-ribo duplexes. DNA polymerase I of *Escherichia coli* was also found to use the hybrid template with high efficiency, copying preferentially the ribo strand. With the chicken enzyme, the template activity of denatured DNA was increased tenfold by simultaneous transcription with RNA polymerase. DNA polymerase activity reaches a maximum in the 6- to 8-day chicken embryo and then declines progressively to about one-third of the maximal value in the adult chicken.**

Whether in the course of cellular differentiation primordial biological information is merely uncovered gradually or whether additional information is generated by other mechanisms that are as yet little understood may be considered an open question. We are inclined to prefer the second possibility.

The problems of the conservation and the transfer of biological information can be formulated most easily by postulating the existence of synthetic enzyme systems operating under the guidance of sequence-specific templates. It is, in fact, this question of the template requirements of the nucleic acid polymerases that is at present undergoing a radical reevaluation. The concept of a unidirectional flow of biological information, going always from DNA to RNA to protein (1), has, it would seem, been invalidated by the discovery, first in RNA-containing tumor viruses, of RNA-directed DNA polymerases (2–5).

It did not seem improbable to us that embryonic development may be one of the events during which agents of additional biological information would be called upon to operate; we have for some time been interested in screening the template requirements of the polymerizing enzymes of the growing chicken embryo. This is a preliminary report on a DNA polymerase found in rather high concentration in the early embryos: an enzyme that seems to prefer, as a template, the ribo strand of a synthetic deoxyribo–ribo polymer.

Abbreviation: CMPS, *p*-chloromercuriphenylsulfonate.

* This paper is part I of a series.

MATERIALS AND METHODS

Fertilized hen's eggs (White Leghorn), incubated for varying periods, were supplied by the Shamrock Poultry Farm, North Brunswick, N.J. The embryos were removed from the eggs, freed from membranes and external fluid, washed twice in 0.5 M sucrose solution, and ground with three parts (w/v) of a solution of 0.5 M sucrose (free of ribonuclease) and 0.05 M glycyl-glycine (pH 7.5) in a glass–Teflon tissue grinder (clearance, 0.015–0.023 cm). All operations were performed at 2–4°C. Centrifugation of the mixtures (14,500 × *g*, 30 min) yielded the extracts which served for the enzyme assays.

DNA polymerase I (EC 2.7.7.7) was prepared from *Escherichia coli* according to published procedures (6, 7). The preparation (corresponding to fraction 6 of ref. 7) had a specific activity of 9200 units/mg of protein.

RNA polymerase (EC 2.7.7.6) of very high purity was isolated from *E. coli* by a modification of a recent procedure (*8*). Its absorbance ratio at 280/260 was 1.82; when assayed with intact calf-thymus DNA (10 min, 37°C) the incorporation of CTP amounted to 825 nmol/mg protein. Terminal deoxynucleotidyl transferase was prepared from calf thymus (9); it retained a trace of DNA polymerase activity. Ribonuclease (EC 2.7.7.16) and deoxyribonuclease I (EC 3.1.4.5) were supplied by Worthington Biochemical Corp.

Calf-thymus DNA was isolated in the usual manner (10) and denatured by being kept at 100°C for 10 min. "Activated" DNA was prepared from calf-thymus DNA as described (6).

The synthetic ribo homopolymers poly(rA), poly(rU), poly(rI) and poly(rC), as well as poly(dA) and poly(dT), and the hybrid poly(rA)·poly(dT) were supplied by Miles Laboratories. The unlabeled deoxy- and ribonucleoside triphosphates, as well as the ^3H-labeled deoxyribotriphosphates and the ^{14}C-labeled ribotriphosphates were furnished by Schwarz/Mann, except [^3H]GTP, which came from Amersham/Searle.

RESULTS

Survey of polymerase activities

Extracts of 4-day-old chicken embryos were surveyed for their polymerizing activities with the use of a variety of templates and with both ribo- and deoxyribonucleoside triphosphates. A selection of results is presented in Table 1. This survey permits the preliminary conclusion that the

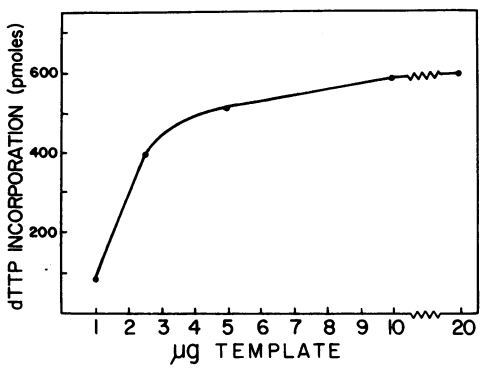

FIG. 1. Action of hybrid-dependent DNA polymerase of 4-day chicken embryo as a function of template concentration. The incubation mixtures contained, in a final volume of 0.13 ml, 0.05 M Tris·HCl (pH 8.3); 1 mM $MnCl_2$; 0.12 M KCl; 20 nmol of [^{3}H]dTTP (40 cpm/pmol); the indicated amounts of poly(rA)·poly(dT); 20 μl of enzyme. Other conditions as in Table 1.

polymerase activity most evident in the embryonic extract is a hybrid-dependent DNA polymerase which is guided by the ribo strand of the template poly(rA)·poly(dT). It will be remembered that this template has been used repeatedly in studies of DNA- and RNA-dependent DNA polymerases (compare refs. 12–14). In contrast, the all-ribo template poly(rA)·poly(rU) showed little activity. There was also some indication of a DNA-dependent RNA polymerase preferring an intact DNA template. A DNA-directed DNA polymerase, on the other hand, was not much in evidence.

Template and metal requirements of hybrid-dependent DNA polymerase

The incorporation of dTTP into a polymer, which is catalyzed by the embryonic extract, requires the presence of a suitable template and rises in measure with its concentration until a plateau is reached (Fig. 1). The activity is not lost upon freezing and thawing, nor is it diminished significantly on prolonged storage at 0°C.

The effects of metal ions on the hybrid-directed dTTP polymerization are as follows: Mg, Zn, or Co ions do not activate the reaction, but Mn ions do so very significantly, the highest incorporation being observed with 1 mM Mn^{2+} together with 0.12 M KCl. If Mg^{2+} also is added, the incorporation of the nucleotides is depressed.

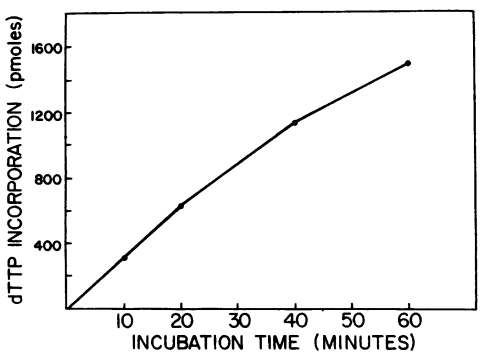

FIG. 2. Action of hybrid-dependent DNA polymerase of 4-day chicken embryo as a function of time. The incubation mixtures contained, in a final volume of 0.6 ml, 0.05 M Tris·HCl (pH 8.3); 1 mM $MnCl_2$; 0.12 M KCl; 60 μg poly(rA)·poly(dT); 120 nmol [^{3}H]dTTP (80 cpm/pmol); and 60 μl of enzyme. Samples of 100 μl were withdrawn at the indicated times and processed as in Table 1.

Under optimum conditions, the polymerization of dTTP proceeds almost linearly for at least 1 hr (Fig. 2). With very long incubation times the uptake drops, probably owing to the presence of traces of a nuclease acting on the polymerized product.

Effect of nucleases

As is shown in Table 2, RNase, whether added together with the enzyme or 20 min later, is without effect on the incorporation of dTTP. DNase, tested under the same conditions, prevented the formation of the enzymic product or degraded it, once formed. Alkali, which hydrolyzes the polyadenylate moiety of the hybrid template, had little effect on the product that had been synthesized before the addition of NaOH. There can be little doubt that the product of enzyme action is polydeoxythymidylate.

Efficiency of different templates; inhibition experiments

Experiments with a variety of base-paired homopolymer duplexes are summarized in Table 3. The most efficient

TABLE 1. *Polymerase activities of extracts of 4-day chicken embryos in the presence of various templates*

Expt. no.	Template	Unlabeled nucleoside triphosphates	Labeled nucleoside triphosphates	Incorporation, (pmol)
1	None	4 ribo	CTP	80
2	None	4 deoxyribo	dTTP	5
3	Calf-thymus DNA, intact	4 ribo	CTP	232
4	Calf-thymus DNA, intact	4 deoxyribo	dTTP	12
5	Calf-thymus DNA, denatured	4 ribo	CTP	117
6	Calf-thymus DNA, denatured	4 deoxyribo	dTTP	32
7	poly(rA)·poly(dT)	dTTP	dTTP	640
8	poly(rA)·poly(dT)	dATP	dATP	47
9	poly(rA)·poly(dT)	UTP	UTP	100
10	poly(rA)·poly(dT)	ATP	ATP	157
11	poly(rA)·poly(rU)	dTTP	dTTP	15
12	poly(rA)·poly(rU)	dATP	dATP	38

The incubation mixtures contained, in a final volume of 0.25 ml, 0.05 M Tris·HCl (pH 8.3); 10 mM $MgCl_2$; 5 mM $MnCl_2$; 10 mM dithiothreitol; 0.04 M KCl; either 40 nmol of each of the indicated deoxyribonucleoside triphosphates or 120 nmol of each ribonucleoside triphosphate; the specified template [40 μg of DNA or 20 μg of either poly(rA)·poly(dT) or poly(rA)·poly(rU)]; and 100 μl of enzyme. The specific activities of the labeled precursors were: [^{3}H]dATP and [^{3}H]dTTP, 80 cpm/pmol; [^{14}C]ATP, [^{14}C]CTP, [^{14}C]UTP, 1700 cpm/nmol. After incubation (37°C, 10 min), the reaction was stopped with 2 ml of 10% trichloracetic acid, and the precipitates were collected and washed on membrane filters (Schleicher & Schuell, type B-6) and counted, in a Beckman scintillation counter, in a dioxane–naphthalene mixture (11). The results were corrected for the background counts (100 cpm for ^{3}H, 40 cpm for ^{14}C). In the third column, listing the unlabeled precursors, "4 ribo" means that ATP, GTP, CTP, and UTP were present and "4 deoxyribo" that dATP, dGTP, dCTP, and dTTP had been added. Incorporation in the last column refers to the uptake of only the labeled precursor.

TABLE 2. *Nature of enzymic product*

Expt. no.	Addition	Time of addition, min after start of incubation	Total incubation time (min)	Incorporation of dTTP into product, (pmol)
1	None	—	20	696
2	None	—	80	2417
3	Ribonuclease	0	20	687
4	Ribonuclease	20	80	2237
5	Deoxyribonuclease	0	20	7
6	Deoxyribonuclease	20	80	39
7	NaOH	20	80	562

The incubation mixtures contained, in a final volume of 0.13 ml, 0.05 M Tris·HCl (pH 8.3); 1 mM $MnCl_2$; 0.12 M KCl; 20 nmol [^{3}H]dTTP (80 cpm/pmol); 10 μg poly(rA)·poly(dT); and 20 μl of enzyme. Where specified, 2 μg of ribonuclease or deoxyribonuclease was added. In Expt. 7, 6 N NaOH was added to a concentration of 1 N NaOH. Other conditions as in Table 1.

template is the hybrid poly(rA)·poly(dT), which directs the incorporation of the nucleotide complementary to its ribo strand, namely, dTTP; the uptake of dATP is minimal. The counterpart hybrid poly(rU)·poly(dA) shows the expected opposite activity, although the preponderance of dATP incorporation is less marked. The all-deoxy duplex, poly(dA)·poly(dT), also directs the uptake of dATP preferentially. The all-ribo duplexes are essentially inactive, with the possible exception of the incorporation of dCTP in the presence of poly(rI)·poly(rC). This is, however, most likely due to a different enzyme.

It was of interest to find that the hybrid-dependent DNA polymerase studied here is—in this respect reminiscent of DNA polymerase II of *E. coli* (15)—strongly inhibited by *p*-chloromercuriphenylsulfonate (CMPS). As can be seen in Table 3, the hybrid-directed incorporation of dTTP (Expt. 3) and of dATP (Expt. 8) is completely abolished.

TABLE 3. *Template efficiency of different polynucleotide duplexes; effect of a mercurial inhibitor*

Expt. no.	Template	Labeled nucleoside triphosphate	Incorporation (pmol) Without inhibitor	Incorporation (pmol) With CMPS
1	None	dTTP	20	17
2		dATP	29	28
3	poly(rA)·poly(dT)	dTTP	663	22
4		dATP	44	28
5	poly(dA)·poly(dT)	dTTP	42	21
6		dATP	242	37
7	poly(rU)·poly(dA)	dTTP	27	20
8		dATP	72	35
9	poly(rA)·poly(rU)	dTTP	19	21
10		dATP	34	29
11	poly(rI)·poly(rC)	dCTP	52	49
12		dGTP	29	34

The incubation mixture contained, in a final volume of 0.13 ml, 0.05 M Tris.HCl (pH 8.3); 1 mM $MnCl_2$; 0.12 M KCl; 10 μg of the specified template; 20 nmol of the specified ^{3}H-labeled deoxynucleoside triphosphate (80 cpm/pmol); and 20 μl of the chicken-embryo enzyme. With the exception of poly(rA)·poly(dT), the templates were prepared by mixing equimolar quantities of the two respective homopolymers in 0.2 M NaCl and leaving the mixture at room temperature for 15 min. When the mixtures were kept under annealing conditions (65°C, 2–3 hr) and then cooled slowly, no significant effect on template activity was observed. For treatment with *p*-chloromercuriphenylsulfonate (CMPS), the enzyme was incubated with 1 mM CMPS for 25 min at 35°C before being added to the assay mixtures. Other conditions as in Table 1.

Comparison with other polymerases

The rather unusual properties of the hybrid-dependent polymerase discussed here made it interesting to compare its behavior with that of two other enzymes, namely, the much-investigated DNA polymerase I of *E. coli* and the terminal deoxynucleotidyl transferase of calf thymus. The results are

TABLE 4. *Comparison of chicken-embryo enzyme with other DNA polymerases*

Expt. no.	Enzyme	Template	Unlabeled nucleoside triphosphates	Labeled nucleoside triphosphates	Incorporation (pmol) Without inhibitor	Incorporation (pmol) With CMPS
1	4-day chicken embryo extract	Poly(rA)·poly(dT)	dTTP	dTTP	2385	81
2		Poly(rA)·poly(dT)	dATP	dATP	265	173
3		"Activated" DNA	4 deoxyribo	dTTP	113	57
4	DNA polymerase I of *E. coli*	poly(rA)·poly(dT)	dTTP	dTTP	4119	4193
5		poly(rA)·poly(dT)	dATP	dATP	133	140
6		"Activated" DNA	4 deoxyribo	dTTP	161	192
7	Terminal transferase	poly(rA)·poly(dT)	dTTP	dTTP	276	—

The incubation mixture contained, in a final volume of 0.3 ml, 0.05 M Tris·HCl (pH 8.3); 1 mM $MnCl_2$; 0.12 M KCl; 10 μg of the specified template; 20 nmol of the specified ^{3}H-labeled deoxynucleoside triphosphates (20 cpm/pmol); and 20 μl of the chicken-embryo enzyme or 2.4 pg of DNA polymerase I or 1.6 μg of terminal deoxynucleotidyl transferase. For the treatment with CMPS, see Table 3; for other conditions, see Table 1.

summarized in Table 4. The chicken enzyme behaved as described before in this paper. What was, however, most surprising was the observation that under our conditions of assay (presence of Mn^{2+}, absence of Mg^{2+}), highly purified DNA polymerase I exhibited the same template preferences: it barely utilized "activated" DNA, which is its normal template when acting in the presence of Mg^{2+} (6), but was very effective when confronted with the hybrid template poly(rA)·poly(dT). The two enzymes differed, however, in their sensitivity to the Hg inhibitor: the *E. coli* enzyme was not inhibited by CMPS. The fact that also with DNA polymerase I the incorporation, in the presence of poly(rA)·poly(dT), concerned mostly dTTP, that of dATP amounting only to 3% of the uptake of dTTP, would again seem to point to guidance by the ribo strand of the hybrid template, but other explanations cannot yet be excluded, and these observations will form the subject of a separate study. The terminal transferase, on the other hand, scarcely utilized the hybrid, which renders a primer function of the template rather unlikely.

Effect of DNA transcription on action of hybrid-dependent DNA polymerase

The most cogent demonstration that the chicken embryo enzyme does prefer a hybrid template is provided by the experiments in Table 5, in which the cooperation between transcribing and replicating conditions, with denatured calf-thymus DNA as the template, is brought out clearly. The cofactor effect of Mn^{2+} is again emphasized. The synergism of the DNA and RNA polymerases in the presence of precursors of both DNA and RNA is underlined by the comparison of Expt. 1 with Expt. 2 to 4. Rifampicin inhibited the polymerization to the same extent as did the omission of RNA polymerase.

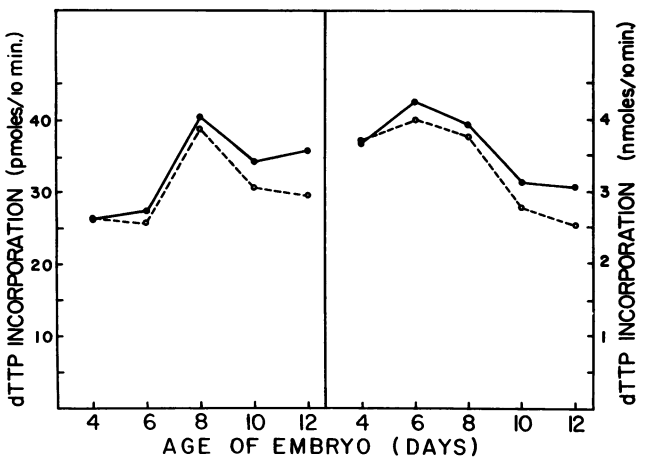

FIG. 3. Variations of enzyme activity with embryonic age. The incubation mixtures contained, in a final volume of 0.13 ml, 0.05 M Tris·HCl (pH 8.3); 1 mM $MnCl_2$; 0.12 M KCl; 20 nmol of [^{3}H]dTTP (80 cpm/pmol); 10 μg of poly(rA)·poly(dT); and 20 μl of the enzyme solution corresponding to the indicated embryonic stage. The average wet weights of the embryos were: 4-day, 50 mg; 6-day, 450 mg; 8-day, 1.1 g; 10-day, 2.5 g; 12-day, 5 g. The protein concentration of the extracts was, in the same respective order: 3.57, 3.23, 5.13, 5.50, and 5.83 mg/ml. Other conditions as in Table 1. *A.* Incorporation of dTTP as pmol/mg of wet tissue. *B.* Incorporation of dTTP as pmol/mg of protein in extract. *Solid lines*, incubation for 10 min; *broken lines*, one-half of the values yielded by incubation for 20 min.

Variations of enzyme activity with embryonic age

We show, in Fig. 3, the results of a comparative study of the levels of hybrid-dependent DNA polymerase in embryos of 4–12 days. The values are expressed as activity per weight of embryonic tissue and also per mg of protein; they suggest a maximum at 8 days in the first case, and at 6 days in the second. Up to the stage of 8 days the incorporation of dTTP proceeds in a nearly linear fashion, so that the uptake of precursor within 20 min is exactly twice that recorded in 10 min. In later stages the curves begin to diverge, probably due to the appearance, at that time, of nucleases degrading the enzymic product.

Preliminary experiments on the concentration of this enzyme in the liver, brain, and heart of 20-day-old embryos and of adult animals have demonstrated the existence of a similar activity at progressively lower levels: at 20 days it is, in these organs, about one-half of the activity of the 4-day embryo extract, in adults it is about one-third. The organs examined did not differ significantly in enzyme concentration.

CONCLUDING REMARKS

Science progresses often from oversimplification to overgeneralization and finally, in a few cases, to truth. As regards the mechanisms through which the cell achieves the synthesis of the complex sequence-specific heteropolymers DNA and RNA, it must be admitted that we have barely gone beyond the first stage. At present, at least two different DNA polymerases have been described in *E. coli* which, under specific conditions, utilize DNA as the template for the alignment of the nucleotide precursors (6, 15, 16). Other DNA polymerases

TABLE 5. *Simultaneous transcription by RNA polymerase and replication by hybrid-dependent DNA polymerase of denatured calf-thymus DNA*

Expt. no.	Conditions	Incorporation of dTTP, (pmol) with Mn^{2+}	with Mg^{2+}
1	Complete system*	661	214
2	Complete system, staggered†	1030	—
3	Without RNA polymerase	63	116
4	Without chicken-embryo enzyme	15	16
5	Without ribonucleoside triphosphates	111	52
6	Rifampicin (4 μg/ml) added to complete system	64	121

The complete system contained, in a final volume of 0.13 ml, 0.05 M Tris·HCl (pH 8.3); 1 mM $MnCl_2$ or 7 mM $MgCl_2$; 0.12 M KCl; 5 μg of heat-denatured calf-thymus DNA; 5 nmol each of the 4 deoxyribonucleoside triphosphates, with [^{3}H]dTTP (32 cpm/pmol); 60 nmol (each) of the 4 ribonucleoside triphosphates; 3 μg of RNA polymerase; 20 μl of the 4-day chicken-embryo enzyme. Incubation, 1 hr at 37°C; other conditions as in Table 1.

* When the ribo precursors were labeled with [^{14}C]CTP, approximately 600 pmol of this nucleotide were incorporated in the presence of Mn^{2+} and about 80 pmol in the presence of Mg^{2+}.

† The complete system minus chicken-embryo enzyme was incubated for 1 hr, then this enzyme was added and incubation was continued for another hr.

prefer RNA templates (2–5, 14). Similarly, DNA-dependent RNA polymerases have been much studied (17) and, more recently, also RNA polymerases that utilize RNA as the template (18). We suggest that the enzyme from chicken embryo studied may belong to yet another class of DNA polymerases—perhaps similar to those found in mammalian cells (14)—which are guided by the ribo strand of a hybrid template.

It must, however, be understood that the template requirements, as they are ascertained under the controlled conditions of an *in vitro* assay, are not necessarily absolute. Thus, DNA polymerase I of *E. coli* has been reported to be able to use a synthetic all-ribo duplex as template (19), and we have shown here in Table 4 that the same enzyme, supplemented with Mn^{2+} instead of Mg^{2+}, is guided by the ribo strand of a synthetic hybrid. In a similar fashion, the DNA-dependent RNA polymerase can also accept RNA as a template (compare, e.g., ref. 20).

Certain nucleic acid polymerases, e.g., the microbial RNA polymerases, seem to exhibit a very broad spectrum of template acceptance, whereas others, e.g., $Q\beta$ "replicase", are extremely specific in their requirements. There may also be cases in which the latter type is superposed upon the former, e.g., in the transformation of an animal cell by a virus; and in the absence of much more refined methods than are available, great caution must be exercised in drawing diagnostic conclusions from changes in template requirements. The regulation of replication and transcription in a tissue cell must be a highly ordered process with which a whole series of enzymes, possibly integrated into a complex structure, may be concerned; and it will not be easy to correlate what is learned *in vitro* about the behavior of separated polymerases with what happens in the living cell.

The suggestion that the DNA polymerase of the chicken embryos is a hybrid-dependent enzyme which is directed by the ribo strand of the template will have to be corroborated by additional experiments, which are being undertaken. It is, for instance, not yet clear whether the RNA portion synthesized by RNA polymerase (Table 5) acts as a template, as a primer, or as both for the simultaneous action of DNA polymerase, nor what the real function of the polyribo strand of a synthetic hybrid duplex is. (For the distinction between primer and template functions we refer to a previous discussion in ref. 21.)

It would obviously be of great biological interest if the existence of such hybrid-dependent DNA polymerases could be put on a firm basis, but we should like to postpone a detailed discussion until we know more.

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